BIOCELL
2022 46(10): 2225-2229

Tech Science Press

A nanostructured look of collagen apatite porosity into human mineralized collagen fibril

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Key words: Bone nanostructure, Diffusivity, Biomimicking, Bioscaffolds, Tissue engineering

Abstract: Bone tissue is a hierarchical material characterized at nanoscale by the mineralized collagen fibril, a recurring structure mainly composed of apatite minerals, collagen and water. Bone nanostructure has a fundamental role in determining the mechanical behavior of the tissue and its mass transport properties. Diffusion phenomenon allows to maintain an adequate supply of metabolites in the mechanisms of bone remodeling, adaptation and repair. Several analytical and computational models have been developed to analyze and predict bone tissue behavior. However, the fine replication of the natural tissue still represents a challenge. Insights on the structural organization at nanoscale and on the influence of apatite mineral crystals on the diffusion coefficient lead to outline the functional conditions for the development of biomimetic strategies for bone tissue engineering. Thorough understanding of bone nanostructure is essential to improve longevity of bioscaffolds and to decrease the risk of failure by controlling their mechanical and biological performance.

Introduction

Many natural biological tissues are organized in hierarchical structures spanning multiple length scales. This strategy provides improved properties of tissues with respect to those of individual components. For instance, bone is a complex mineralized connective tissue that displays hierarchical structure, from nano- to macro-scale (Reznikov *et al.*, 2018). At nanoscale, it is composed of apatite minerals, collagen, water and a modest percentage of non-collagenous proteins and proteoglycans. In the last decade, studies (Wang *et al.*, 2013; Bertolotti *et al.*, 2021) highlighted that apatite platelets are encapsulated in a core-shell structure by a hydrated amorphous calcium phosphate layer that provides favorable chemical environment for ion exchange and for the interaction between minerals. The combination of the brittle mineral phase with the ductile collagen matrix and their arrangement in periodic structures, i.e., building blocks, at critical length scales allow high strength and toughness, maximizing functionality and minimizing weight and energy cost (Giesa and Buehler, 2013; Wegst *et al.*, 2015).

Studies concerning the influence of the hierarchical structure on tissue properties facilitate better understanding of the effect of aging, pathologies and treatments (Gao, 2006) and may help developing new standard care based on tissue engineering. In this sense, the present viewpoint addresses the advancement associated with the diffusion analysis in the nanoconfined structures of the mineralized collagen fibril (MCF). The understanding of nanoconfinement features and their influence on diffusion process may provide rewarding opportunities in numerous research areas as bone tissue engineering, regenerative medicine, nanomechanics and nanofluidics.

Viewpoint

Fluid flow is essential for bone vitality. Specifically, molecular transport is necessary to maintain an adequate supply of nutrients, growth factors, mineral ions and solutes employed in the mechanisms of bone remodeling, adaptation and repair. In bone tissue, the metabolic traffic and interchange of signaling molecules, physiological solutes and fluids are strongly dependent on the transport pathways comprising, at the smallest hierarchical structural level, the interconnected pores within the collagen-apatite matrix (Cowin, 2001). Water mediates the interaction between apatite minerals and collagen (Wang *et al.*, 2013) and its presence also influences bone stiffness and stress (Maghsoudi-Ganjeh *et al.*, 2020). It is commonly accepted that bone presents four levels of

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Received: 01 January 2022; Accepted: 18 March 2022

Doi: 10.32604/biocell.2022.021150

porosity, which are nested hierarchically one inside another: collagen-apatite (10 nm), canalicular (100 nm), lacunar (up to 8 μm), vascular (50 μm), and intertrabecular porosity (up to 1 mm) (Cowin, 2001).

The role of the fluid phase in bone tissue has been thoroughly analyzed, especially at the lacunar-canalicular porosity levels (Cowin, 2001; Fritton and Weinbaum, 2009). Recently, an experimental and computational study of van Tol *et al.* (2020) highlighted the influence of fluid flow within the lacunar-canalicular architecture on the feedback loop of bone remodelling. Nonetheless, relatively little is known about the properties of the spaces through which fluid flows at bone nanostructure. Despite the paucity of experimental information, some studies have postulated the mechanism of flow within bone nanostructure and its role in physiological and pathological conditions. An experimental analysis performed by Marinozzi and colleagues (Marinozzi *et al.*, 2014a; 2014b) investigated water diffusion within a single trabecula. The study (Marinozzi *et al.*, 2014a) provided new insights up to the length scale of bone principal constituents, i.e., collagen matrix and apatite mineral. An air-dried single trabecula from human femur head was completely immersed in water and displacements along the three main axes (Length L, Width W and Thickness T) of the plate-like trabecula were measured with a high accuracy dilatometer. Analysis of the swelling over time along the three axes of the trabecula led to information concerning water diffusion from external surfaces to the internal structure of the specimen. The hygroexpansion of the trabecula sample appeared sensitive essentially to the water transport at the collagen–apatite length scale.

Subsequently, a 3D analytical model of the water uptake (Marinozzi *et al.*, 2014b) was developed to predict the diffusion coefficients along the three axes of the trabecula by means of a genetic algorithm. The major diffusivity was in the longitudinal direction, i.e., $D_L = 1.03 \cdot 10^{-9}$ $m^2 \cdot s^{-1}$, while minor values, with one and two orders of magnitude than D_L, corresponded to D_W, i.e., $D_W = 1.26 \cdot 10^{-10}$ $m^2 \cdot s^{-1}$ and respectively D_T, i.e., $D_T = 1.16 \cdot 10^{-11}$ $m^2 \cdot s^{-1}$. These studies highlighted that water flow is observed also in conditions characterized by small pore size, e.g., roughly 2 nm (Lemaire *et al.*, 2015), i.e., when confinement effect is significant. Furthermore, the water flow at nanoscale and the anisotropic behavior of the diffusion coefficient were confirmed also by molecular dynamics studies (Lemaire *et al.*, 2015; di Tommaso *et al.*, 2017).

Difficulty in performing experimental measurements at bone nanoscale led to the implementation of complementary strategies, as numerical modelling. Computational studies based on Monte Carlo technique investigated the influence of mineral arrangement (Bini *et al.*, 2017) and the effect of structural factors (Bini *et al.*, 2019a), e.g., tortuosity and constrictivity, on the diffusivity within bone nanostructure. The methodology presented in the works of Bini *et al.* (2017; 2019a) allowed to provide quantitative information regarding mineral inclination. It is worth pointing out that the predicted apatite orientation obtained from the 3D computational model, i.e., in a range of ± 20 degrees, finds agreement with subsequent experimental investigations that utilized high resolution tomographic techniques (Xu *et al.*, 2020). In a recent computational model (Bini *et al.*, 2021a), diffusion behavior was analyzed also in relation to the variation of bone mineral content. The diffusion phenomenon of water in the MCF was modelled as a 3D random walk process. Obviously, the structural alterations observed for low mineral content are associated with an increase of the water diffusion. Conversely, hypermineralized conditions are characterized by lower values of diffusivity.

A computational model (Bini *et al.*, 2021b) also investigated the effect of the mineral volume fraction on the organization of bone nanostructure applying the percolation theory, which is a research field of statistical physics that studies the connectivity in a system. The percolation phenomenon is characterized by the onset of connected networks that span the domain of interest from one side to another. The most studied situations are those in proximity of the appearance of overall connectivity, i.e., at percolation threshold, since it represents a phase transition that leads to changes in the mechanical, chemical or biological properties of the structure. Applying the Monte Carlo technique, (Bini *et al.*, 2021b) investigated the formation of spanning networks of apatite crystals for different mineral volume fractions characteristic of human bone tissue. The outcomes of the study of Bini *et al.* (2021b) highlighted an increase of the groups of connected apatite platelets in hypermineralized collagen fibrils. The onset of spanning clusters of minerals is also consistent with recent experimental investigations of Reznikov *et al.* (2018) and Xu *et al.* (2020). Moreover, previous studies (Landis *et al.*, 1993; Currey, 1969) suggested the possibility of coalescence of apatite minerals, that conceivably may alter the MCF properties.

The analyses of diffusion and the investigation of the development of connected network of apatite crystals concerned cases of hypo– and hyper–mineralization that could reflect pathological conditions. In experimental studies (Faibish *et al.*, 2005; Milovanovic *et al.*, 2011; Parle *et al.*, 2019), assessment of mineral content and tissue architecture highlighted that aging, diseases of low mineral content (osteomalacia) or high mineralization (osteoporosis) produce abnormalities in bone structure. It is a current research interest of the Authors to analyze the variation of the diffusion phenomenon in presence of percolating clusters of apatite minerals (Fig. 1). It is expected that this abnormal structural organization could highly modify the diffusion behavior at bone nanoscale. In this sense, an analysis of Schurman *et al.* (2021) at the microscale showed that the decrease of canalicular connections due to aging reduces diffusion and leads to deficit of fluid velocity and subsequently to limited mechanostimulation. Consequently, we consider appropriate to investigate how percolating clusters modify diffusion in detriment to cells nutrition, growth and proliferation.

As illustrated, bone properties are closely related to its hierarchical structure. Several analytical and computational models were developed to analyze and predict bone tissue behavior. However, the fine replication of the natural tissue still represents a challenge. Biomaterials play an important role in enhancing tissue regeneration since provide structural support for the new tissue. The ideal scaffold should possess biocompatible and biodegradable material,

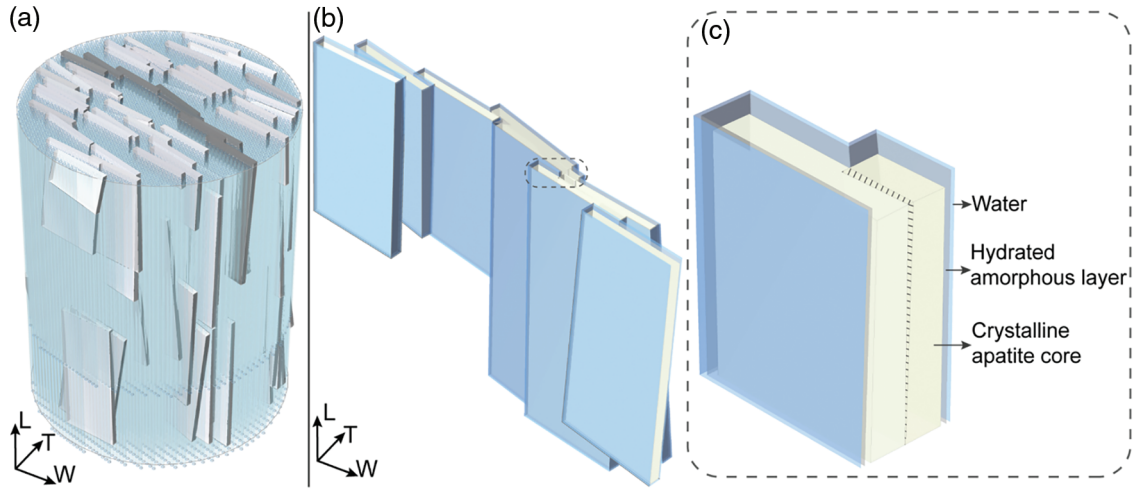

FIGURE 1. Challenge of viewpoint. (a) Section parallel to the equatorial plane, i.e., WT plane, of the 3D model of the mineralized collagen fibril composed of tropocollagen (light blue) and apatite platelets (gray). The dark gray platelets represent a percolating cluster. In (b), an illustration of the unanswered research question that concerns the analysis of the diffusion phenomenon in presence of percolating clusters of minerals. In (c), enlarged view of mineral platelets characterized by a crystalline apatitic core and an amorphous layer composed of (PO_4^{3-}, Ca^{2+}, OH^-, CO_3^{2-}) and (HPO_4^{2-}, Ca^{2+}, CO_3^{2-}, H_2O), respectively. The hatching filled zone represents the interpenetration region of apatite platelets as already investigated in Bini *et al.* (2021b).

suitable porosity, surface area for cell attachment and proliferation and architecture to meet the mechanical demand of the tissue environment (Hajiali *et al.*, 2021; Jurak *et al.*, 2021). Despite the positive achievements presented in literature (Fernandez-Yague *et al.*, 2015), current biomimetic scaffolds have different drawbacks as cell death due to limited nutrient transport, inadequate integration of regenerated tissue with surrounding native tissue and mismatch of scaffold properties with respect to host tissue.

Future Perspectives

Investigations of the structural organization at nanoscale outline the functional conditions that should be considered for the development of biomimetic strategies for tissue engineering and for the creation of biomaterials with similar properties to those of bone tissue. Information concerning the spatial arrangement of minerals (Bini *et al.*, 2017; 2019a; Reznikov *et al.*, 2018; Xu *et al.*, 2020) and development of rapid *in vitro* process of mineralization inside collagen fibrils.

Fang *et al.* (2021) may facilitate the design of synthetic systems. For instance, the mineralization degree of the scaffold should maintain the strength and toughness of the structure at physiological values. To stimulate cell adhesion, proliferation and differentiation, appropriate conditions for the transport of nutrients, waste products or signaling molecules should be developed also within biomimetic scaffolds. Thus, characteristics of the behavior of confined water within bone nanostructure should be considered in the properties of bioscaffolds. The outcomes of the afore-mentioned studies concerning the influence of minerals on the diffusivity and information about the critical behavior of bone tissue dependent on mineral content, may help to control the mechanical and biological performance of bioscaffolds. Thorough knowledge of bone nanostructure is essential to improve bioscaffolds longevity and to decrease failure risk.

To date, in compliance also with the 3R animal experiment requirements, i.e., reduction, refinement and replacement (Baker *et al.*, 2016), computational studies represent a fundamental strategy for the optimization of scaffold design, enabling prediction of structure properties as a complement to experimental investigations. Implanted bioscaffolds should ensure mechanical stability, appropriate diffusion properties and facilitate infiltration of native tissue cells, e.g., osteoblasts (Collins *et al.*, 2021). Most studies focused on the optimization of mechanical behavior of scaffolds by means of computational studies, i.e., mechanobiology-based optimization algorithm (Percoco *et al.*, 2020) or finite element models (Perier-Metz *et al.*, 2021). Conversely, literature herein considered confirms that the fine tuning of mass transport properties is scarcely developed and strengthen the hypothesis illustrated in this viewpoint. Recently, Nguyen *et al.* (2019) implemented image-based simulations to optimize scaffold parameters and culturing conditions to resolve difficulties with oxygen delivery to cells. Mass transport properties should be considered to ensure cell viability within the scaffold and the appropriate trade-off between improving metabolites delivery to cell and avoiding detaching cells phenomenon due to excessive shear forces generated by fluid flow (Nguyen *et al.*, 2019). Diffusion analysis achieved with computational models may provide information for biomaterials selection and scaffold design with the aim of ensuring functionality also at the interface between scaffold and native bone tissue.

Understanding diffusion phenomenon in nanoconfined structures as MCF imparts the ability to control nanofluidics and paves the way for mimicking diffusion in biological system and enhancing the development of physiological relevant bone scaffolds. Increased aging population emphasizes the need for novel approaches to repair tissue lost through trauma or disease. As discussed, many questions are still open and merit attention in future studies. Application of nanotechnology (Araneo *et al.*, 2015) and computational models (Bini *et al.*, 2019b; 2021c) to regenerative medicine is a rapidly growing area of research. A breakthrough in this field may be represented by the

successful development of scaffold that combines morphological, mechanical and mass transport properties similar to those of bone tissue. The fine replication of natural tissue plays an important role in the field of bone regeneration and treatment of degenerative pathologies, e.g., osteoporosis and osteoarthritis.

Availability of Data and Materials: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Author Contribution: The authors confirm contribution to the paper as follows: conceptualization: F.B., A.M., F.M.; methodology: F.B., A.P., F.M.; software: A.P.; validation: F.B., A.P., A.M., F.M.; formal analysis: A.P.; investigation: F.B., A.P., F.M.; resources: A.P., A.M.; data curation: A.P.; writing–original draft: F.B., A.P.; writing-review and editing: F.B., A.P., A.M., F.M.; visualization: A.P.; supervision: F.B., A.M., F.M. All authors reviewed the results and approved the final version of the manuscript.

Ethics Approval: Not applicable.

Funding Statement: The authors received no specific funding for this study.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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